



# Regulation of the cardiac sodium channel $\text{Na}_v1.5$ by utrophin in dystrophin-deficient mice

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<b>Aims</b>	Duchenne muscular dystrophy (DMD) is a severe striated muscle disease due to the absence of dystrophin. Dystrophin deficiency results in dysfunctional sodium channels and conduction abnormalities in hearts of <i>mdx</i> mice. Disease progression in the <i>mdx</i> mouse only modestly reflects that of DMD patients, possibly due to utrophin up-regulation. Here, we investigated mice deficient in both dystrophin and utrophin [double knockout (DKO)] to assess the role of utrophin in the regulation of the cardiac sodium channel ( $\text{Na}_v1.5$ ) in <i>mdx</i> mice.
<b>Methods and results</b>	Co-immunoprecipitation studies in HEK293 cells showed that utrophin interacts with $\text{Na}_v1.5$ via syntrophin proteins, an interaction abolished by deletion of the PDZ (PSD-95, Dlg, and Zona occludens) domain-binding motif of $\text{Na}_v1.5$ . We also provide evidence for such interaction in mouse heart using $\text{Na}_v1.5$ C-terminus fusion proteins. In hearts of DKO mice, $\text{Na}_v1.5$ protein levels were decreased by $25 \pm 8\%$ , together with a $42 \pm 12\%$ reduction of syntrophins compared with <i>mdx</i> , where utrophin was up-regulated by $52 \pm 9\%$ compared with C57BL/10 control mice. Sodium current was found to be reduced by $41 \pm 5\%$ in DKO cardiomyocytes compared with <i>mdx</i> , representing a loss of $63 \pm 3\%$ when compared with C57BL/10 wild-type control mice. Decreased $\text{Na}_v1.5$ protein and current in DKO were reflected in a significant slowing of $27 \pm 6\%$ of maximal upstroke velocity of the cardiac action potential compared with <i>mdx</i> .
<b>Conclusion</b>	Utrophin plays a central role in the regulation of $\text{Na}_v1.5$ in <i>mdx</i> mice. These findings provide support for therapeutic strategies aimed at overexpressing utrophin in the hopes of reducing cardiac pathology in DMD patients.
<b>Keywords</b>	Duchenne muscular dystrophy • Sodium channel • Utrophin up-regulation • Multi-protein complex

## 1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder affecting 1/3500 males and is currently the most severe dystrophinopathy.<sup>1</sup> The disease results from the absence of dystrophin, a protein mainly expressed in striated muscle cells.<sup>2</sup> The main clinical manifestations of dystrophinopathies are loss of skeletal and cardiac muscle strength and elasticity and respiratory insufficiency.<sup>3</sup> Cardiomyopathies are observed in 90% of dystrophic patients, and 20% die from cardiac complications.<sup>4</sup> Dystrophin, a 427 kDa cytoskeletal protein, plays a major structural role in muscle cells. It links the cytoskeleton to the extracellular matrix through the binding of the N-terminus to actin and the C-terminus to the glycoprotein complex at the sarcolemma, forming a dystrophin multi-protein complex (DMC).<sup>5</sup> The *mdx* mouse, which lacks dystrophin, is the classical animal model of DMD.<sup>6</sup> The phenotype of *mdx* mice, however, does not fully mimic that of dystrophic patients. Contrary to human

carriers of mutations causing full length dystrophin deletions, *mdx* mice have a nearly normal lifespan and display different patterns of distribution and severity of fibrosis.<sup>7</sup> Utrophin, a homologue of dystrophin encoded by an autosomic gene, is up-regulated in dystrophic muscle, which may partially compensate for the lack of dystrophin in *mdx* mice, providing an explanation to their milder phenotype.<sup>8</sup> Similar to dystrophin, utrophin is a member of the DMC and binds to syntrophins,<sup>9</sup> which are adapter proteins between different components of the DMC.<sup>10</sup> Syntrophins have been shown to mediate the link between the cardiac voltage-gated sodium channel  $\text{Na}_v1.5$  and dystrophin.<sup>11,12</sup> The  $\text{Na}_v1.5$  channel plays a key role in the depolarization phase of the cardiac action potential (AP) as well as the propagation of the electrical impulse.<sup>13</sup> The primordial role of  $\text{Na}_v1.5$  for normal cardiac function is emphasized by hundreds of mutations found in its gene (*SCN5A*), which have been linked to cardiac diseases such as type-3 long QT syndrome, Brugada syndrome, and dilated cardiomyopathy.<sup>14</sup> In a previous study,<sup>11</sup> our

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group showed that dystrophin plays an important role in the functional expression of the cardiac sodium channel. Na<sub>v</sub>1.5 protein levels and the sodium current were decreased in *mdx5cv* mice, an alternative mouse strain of *mdx* which also lacks full length dystrophin.

Tinsley *et al.*<sup>15</sup> were the first to suggest a compensatory role of utrophin in the absence of dystrophin. In the present study, we investigated this potential role of utrophin in the regulation of the cardiac sodium channel in *mdx* mice. We first describe a syntrophin-mediated interaction between Na<sub>v</sub>1.5 and utrophin. Next, mice deficient for both dystrophin and utrophin [double knockout (DKO)] were used to assess the role of utrophin in the regulation of Na<sub>v</sub>1.5 in mice lacking dystrophin only (*mdx*). Sodium channel protein levels and sodium current density ( $I_{Na}$ ) were decreased in DKO mice compared with *mdx* mice, which was reflected in a significant slowing of maximal upstroke velocity of the AP. These results reveal that utrophin plays a central role in the regulation of Na<sub>v</sub>1.5 in *mdx* mice and provide additional evidence in support of therapeutic strategies aimed at utrophin up-regulation in DMD patients.

## 2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). All animal handling was performed with the permission from the Cantonal Veterinary Administration and according to the Swiss Federal Animal Protection law.

### 2.1 Animals

C57BL/6Ros-5Cv (*mdx5cv*), *mdx*, and their control C57BL/10 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Control C57BL/6j mice were purchased from Janvier (Le Genest, St Isle, France). *Mdx*/DKO mice were obtained from the group of Pr. K.E. Davies (University of Oxford, UK). Male mice, 8–10 weeks of age, were used in this study.

### 2.2 Western blots

HEK293 cells or homogenized whole mouse heart was lysed in 1.0 mL of lysis buffer [50 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 10% glycerol, 1% Triton, 1 mmol/L EGTA supplemented with 10 mmol/L *N*-ethyl maleimide and protease inhibitors (Roche, Basel, Switzerland)]. Protein concentrations were determined by performing Bradford assays (Coo protein dosage kit; Interchim, Montluçon, France). About 40 µg of protein was loaded onto sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE). Protein transfer was performed using the dry system transfer i-blot from Invitrogen (Basel, Switzerland). Immunoblotting was performed using the snap-id system of Millipore (Zug, Switzerland). Detection was achieved using the LICOR system®, and band intensity was quantified with Odyssey software (LICOR, Lincoln, Nebraska, USA). ‘Edge effects’ observed with high-molecular-weight proteins were verified to avoid modifying quantification by loading samples on different SDS–PAGE gels (homemade or Invitrogen pre-cast gel). Despite the presence of edge effects on some gels, values of quantification were unchanged.

### 2.3 Immunoprecipitation studies

About 1 mg of HEK293 cell lysate was incubated for 2 h at 4°C with the appropriate antibody cross-linked to agarose beads using the direct immunoprecipitation kit from Pierce (Lausanne, Switzerland), according to manufacturer’s instructions. The beads were washed three times with lysis buffer, and the immunoprecipitated fractions were analysed by western blot.

### 2.4 Pull-down assays

About 1 mg of total protein was added to 50 µg of glutathione S-transferase (GST)–Na<sub>v</sub>1.5 C-terminus beads and incubated for 2 h at 4°C. After washing the beads three times with lysis buffer, precipitated proteins were eluted with sampling buffer (Invitrogen) and analysed by western blot.

### 2.5 Membrane preparation

Mouse ventricular tissue was homogenized in homogenization buffer containing 50 mmol/L Tris–HCl, pH = 7.5, 150 mmol/L NaCl, 10 mmol/L *N*-ethyl maleimide and protease inhibitors (Roche). The homogenate was centrifuged for 15 min at 1000 g to pellet debris. The supernatant was collected and centrifuged at 4°C for 45 min at 50 000 g. The pellet was resuspended in the homogenization buffer supplemented with 1% Triton and analysed by western blot.

### 2.6 Isolation of mouse ventricular myocytes

Single cardiomyocytes were isolated according to a modified procedure of established enzymatic methods.<sup>11</sup> See Supplementary material online for details.

### 2.7 INa recordings

The whole-cell configuration of the patch-clamp technique was used to record  $I_{Na}$ , as described previously.<sup>11</sup> Details are available in Supplementary material online.

### 2.8 AP recordings

Trains of APs were recorded in the whole-cell configuration of the patch-clamp technique, elicited at 0.5 Hz with rectangular pulses (5 ms at 125% threshold) in the current-clamp mode. Resting potential, amplitude, maximal upstroke velocity ( $dV/dt$ )<sub>max</sub> and durations at 30, 50, and 90% repolarization were averaged from each sequence of APs. See Supplementary material online for details.

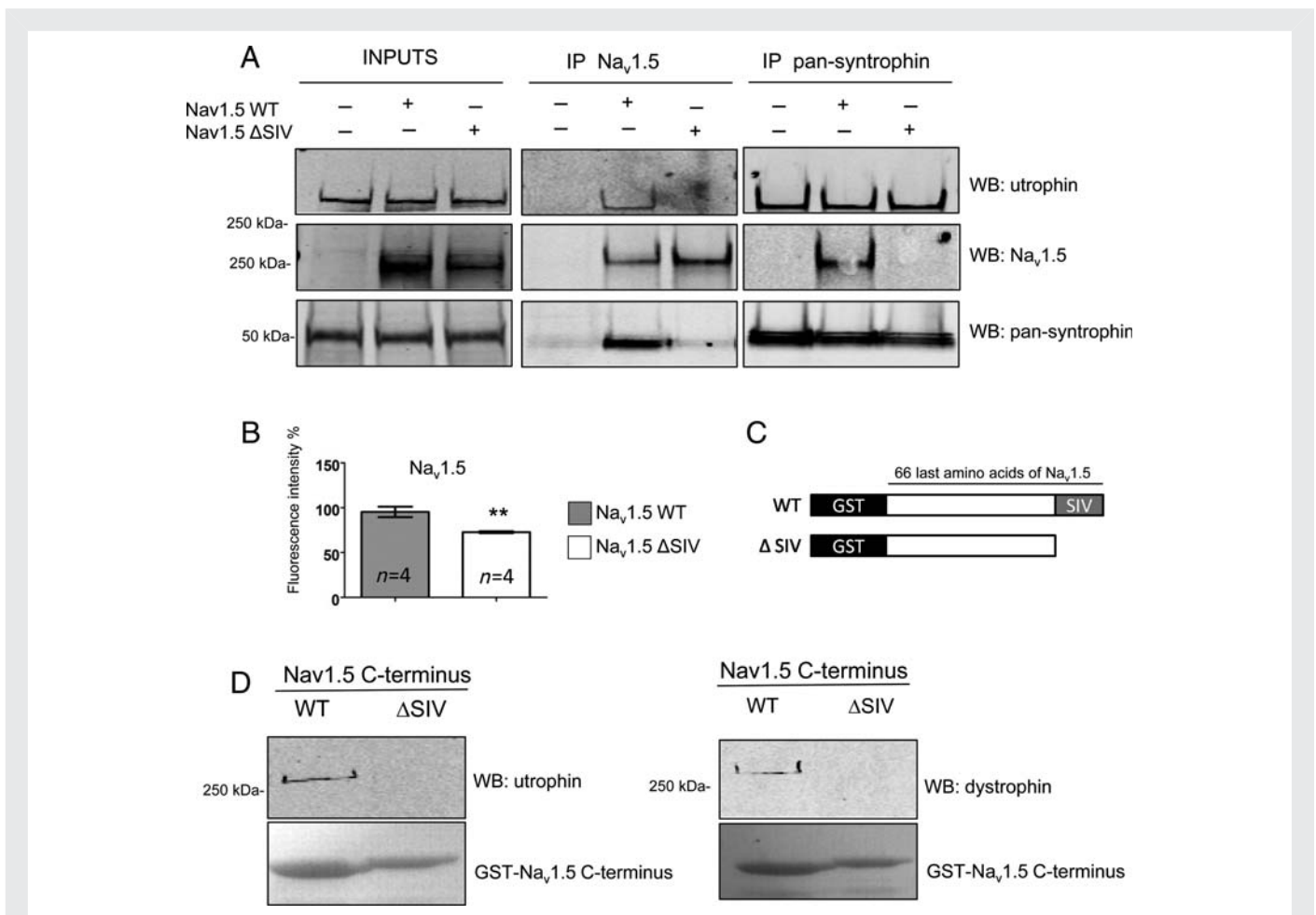
### 2.9 Statistical analyses

Data are represented as mean values ± SEM. Statistical tests applied are described in the legends of the different experiments.

## 3. Results

### 3.1 Na<sub>v</sub>1.5 interacts with utrophin via syntrophin proteins

Na<sub>v</sub>1.5 has been previously shown to interact indirectly with dystrophin and the DMC via syntrophin proteins.<sup>11</sup> The direct interaction between Na<sub>v</sub>1.5 and syntrophins takes place at the last three C-terminal amino acids (SIV) of Na<sub>v</sub>1.5, a PDZ domain-binding motif.<sup>12</sup> Utrophin, a homologue protein of dystrophin, is also present in the DMC<sup>16</sup> and was similarly shown to interact with syntrophins. We used HEK293 cells to investigate whether an interaction between Na<sub>v</sub>1.5 and utrophin exists as they have a robust endogenous expression of utrophin, but not dystrophin (see Supplementary material online, *Figure S1*). HEK293 cells were transiently transfected with wild-type (WT) Na<sub>v</sub>1.5 or Na<sub>v</sub>1.5 ΔSIV, the WT channel with the three last amino acids deleted. Na<sub>v</sub>1.5 was immunoprecipitated, yielding the co-immunoprecipitated fraction with antibodies against syntrophin and utrophin. We found that both syntrophin and utrophin interact with the WT channel (*Figure 1A*) and that these interactions are abolished when Na<sub>v</sub>1.5 ΔSIV is immunoprecipitated. This finding confirms that the interaction occurs via the SIV motif of Na<sub>v</sub>1.5. In addition, utrophin was identified by mass spectrometry in the co-immunoprecipitated fraction of Na<sub>v</sub>1.5 from HEK293 cells (data



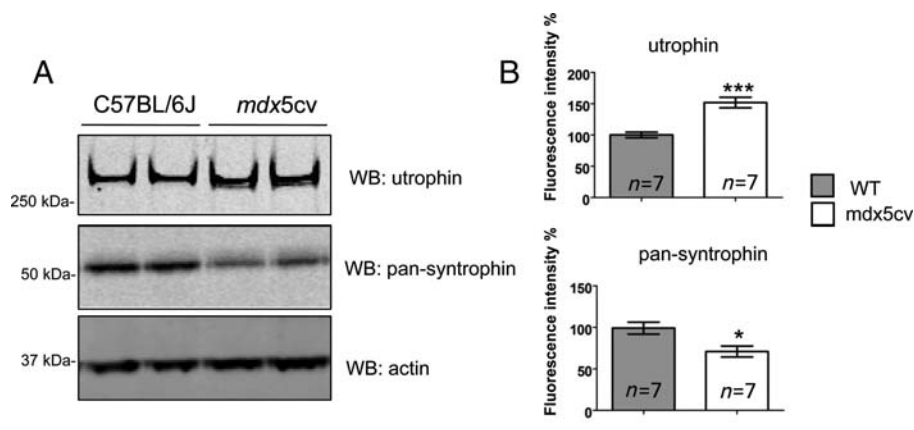
**Figure 1** Na<sub>v</sub>1.5 and utrophin interact indirectly via the direct interaction of the PDZ-domain binding motif (SIV) of the channel and syntrophin proteins. (A) Left panel: input signals confirm expression of utrophin, Na<sub>v</sub>1.5 (WT and ΔSIV), and syntrophins in HEK293 cells. Middle and right panels: syntrophins and utrophin co-immunoprecipitate with WT Na<sub>v</sub>1.5, but not with the mutant channel. (B) Fluorescence intensity of Na<sub>v</sub>1.5 WT and Na<sub>v</sub>1.5 ΔSIV, obtained from four different experiments, was quantified with LICOR Odyssey software (\*\**P* < 0.01; two-tailed Student's *t*-test). (C) Schematic representation of the two fusion proteins used for the pull-down experiments described in (D) comprising the last 66 amino acids of Na<sub>v</sub>1.5 with the PDZ domain-binding motif (SIV). ΔSIV corresponds to the last 63 amino acids carrying the S2014stop mutation. (D) Western blots of pull-down fractions performed on mouse ventricular lysates against utrophin and dystrophin. The bottom panel shows a Ponceau staining of a representative nitrocellulose membrane, showing the presence of GST fusion proteins for different pull-down experiments.

not shown), confirming that the utrophin signal detected with the anti-utrophin antibody is not due to a cross-reaction with a homologous protein, such as dystrophin. Syntrophin proteins were then immunoprecipitated, yielding a co-immunoprecipitated fraction blotted with antibodies against Na<sub>v</sub>1.5 and utrophin. Interactions between utrophin and syntrophins as well as those between syntrophins and Na<sub>v</sub>1.5 were confirmed (Figure 1A). Moreover, the absence of Na<sub>v</sub>1.5 ΔSIV in the co-immunoprecipitated fraction of syntrophins suggests that there is no direct interaction between Na<sub>v</sub>1.5 and utrophin when the SIV sequence of the channel is absent. As shown in Figure 1B, we observed a significant reduction (23 ± 6%) in the total Na<sub>v</sub>1.5 ΔSIV protein level compared with Na<sub>v</sub>1.5 WT, suggesting that the interaction between the channel and syntrophin proteins affects Na<sub>v</sub>1.5 expression in HEK293 cells. To determine whether Na<sub>v</sub>1.5 and utrophin interact in the mouse heart, where both utrophin and dystrophin are expressed, we used GST fusion proteins containing either the last 66 amino acids of the Na<sub>v</sub>1.5 C-terminus or the corresponding Na<sub>v</sub>1.5 C-terminus deleted for the

SIV motif (Figure 1C). The GST fusion proteins were used to pull-down interacting proteins from WT C57BL/6J mouse heart lysates. As shown in Figure 1D, the WT C-terminus of Na<sub>v</sub>1.5 interacts with both dystrophin and utrophin in the mouse heart, and these interactions are SIV motif-dependent. These findings suggest that utrophin, like dystrophin, may be an *in vivo* partner of the cardiac voltage-gated sodium channel Na<sub>v</sub>1.5.

### 3.2 Utrophin is up-regulated in the hearts of mdx5cv dystrophin-deficient mice

Several studies have shown an up-regulation of utrophin in dystrophic skeletal and cardiac muscle of different species, including the human, mouse, and hamster.<sup>17–19</sup> However, to our knowledge, the possible up-regulation of utrophin in cardiac muscle of *mdx5cv* mice had not yet been investigated. We set out to determine whether utrophin is up-regulated in the hearts of *mdx5cv* mice in which the cardiac sodium channel Na<sub>v</sub>1.5 protein and current were shown to be



**Figure 2** Utrophin is up-regulated in the hearts of *mdx5cv* mice. For comparison of the amounts of utrophin as well as syntrophin present in the hearts of control and *mdx5cv* mice, 40  $\mu$ g of ventricular lysates was loaded on SDS–PAGE gels. The protein concentration of each lysate was measured in triplicate by Bradford assays to guarantee equivalent loading and confirmed by Ponceau coloration and western blots against actin. (A) Representative western blots performed with the indicated antibodies. (B) The amount of utrophin and syntrophins examined was assessed by fluorescence intensity quantification with LICOR Odyssey software ( $n = 7$ ; \* $P < 0.05$ , \*\*\* $P < 0.001$ ; two-tailed Student's  $t$ -test).

reduced.<sup>11</sup> Western blot experiments show that *mdx5cv* mice exhibit an up-regulation of utrophin, as illustrated in Figure 2A. A  $52 \pm 9\%$  increase in utrophin expression was found in *mdx5cv* heart lysates (Figure 2B), similar to that found in *mdx* mice compared with C57BL/10 mice.<sup>20</sup> A down-regulation of syntrophin by  $28 \pm 10\%$  was concomitantly observed in *mdx5cv* mice when compared with control mice (Figure 2A and B), consistent with other studies showing that several proteins of the DMC, including syntrophin, were down-regulated in the absence of dystrophin.<sup>21</sup>

### 3.3 Sodium current and Na<sub>v</sub>1.5 protein are down-regulated in the hearts of DKO mice

Given that utrophin may be a partner of Na<sub>v</sub>1.5 and is up-regulated in dystrophin-deficient mice, we assessed the consequence of utrophin deletion in *mdx* mice (DKO) on  $I_{Na}$ . We previously found a 30% reduction in peak sodium current in cardiomyocytes of *mdx5cv* mice.<sup>11</sup> To confirm whether a decrease in  $I_{Na}$  is also present in *mdx* mice, we performed patch-clamp experiments in the whole-cell configuration on freshly isolated cardiomyocytes. As shown in Figure 3A and B,  $I_{Na}$  peak measured at  $-30$  mV is reduced by  $37 \pm 6\%$  in *mdx* cardiomyocytes compared with the control C57BL/10 mice, confirming the common regulatory mechanism of  $I_{Na}$  between these different *mdx* strains. Compared with littermate dystrophin-deficient *mdx* mice, a  $41 \pm 5\%$  reduction in  $I_{Na}$  (Figure 3A and B) was found in DKO mice, representing a  $63 \pm 3\%$  decrease in  $I_{Na}$  when compared with C57BL/10 control mice (Figure 3A and B). The difference in the peak  $I_{Na}$  between the three groups was accompanied by small but statistically significant changes in the voltage dependence of activation and steady-state inactivation (Figure 3C and Table 1). Quantification of Na<sub>v</sub>1.5 protein levels by western blot in ventricular lysates of DKO and *mdx* mice revealed a statistically significant decrease in Na<sub>v</sub>1.5 ( $25 \pm 8\%$ ) in DKO mice compared with *mdx* littermates (Figure 4A and B), providing a potential explanation for the decrease in  $I_{Na}$ . The amount of sodium channel proteins was also compared in membrane fractions from C57BL/10, *mdx*, and DKO hearts (Figure 4C). These experiments confirmed the statistically significant decrease in Na<sub>v</sub>1.5 protein levels between C57BL/10 and *mdx* ( $17 \pm 4\%$ ) and

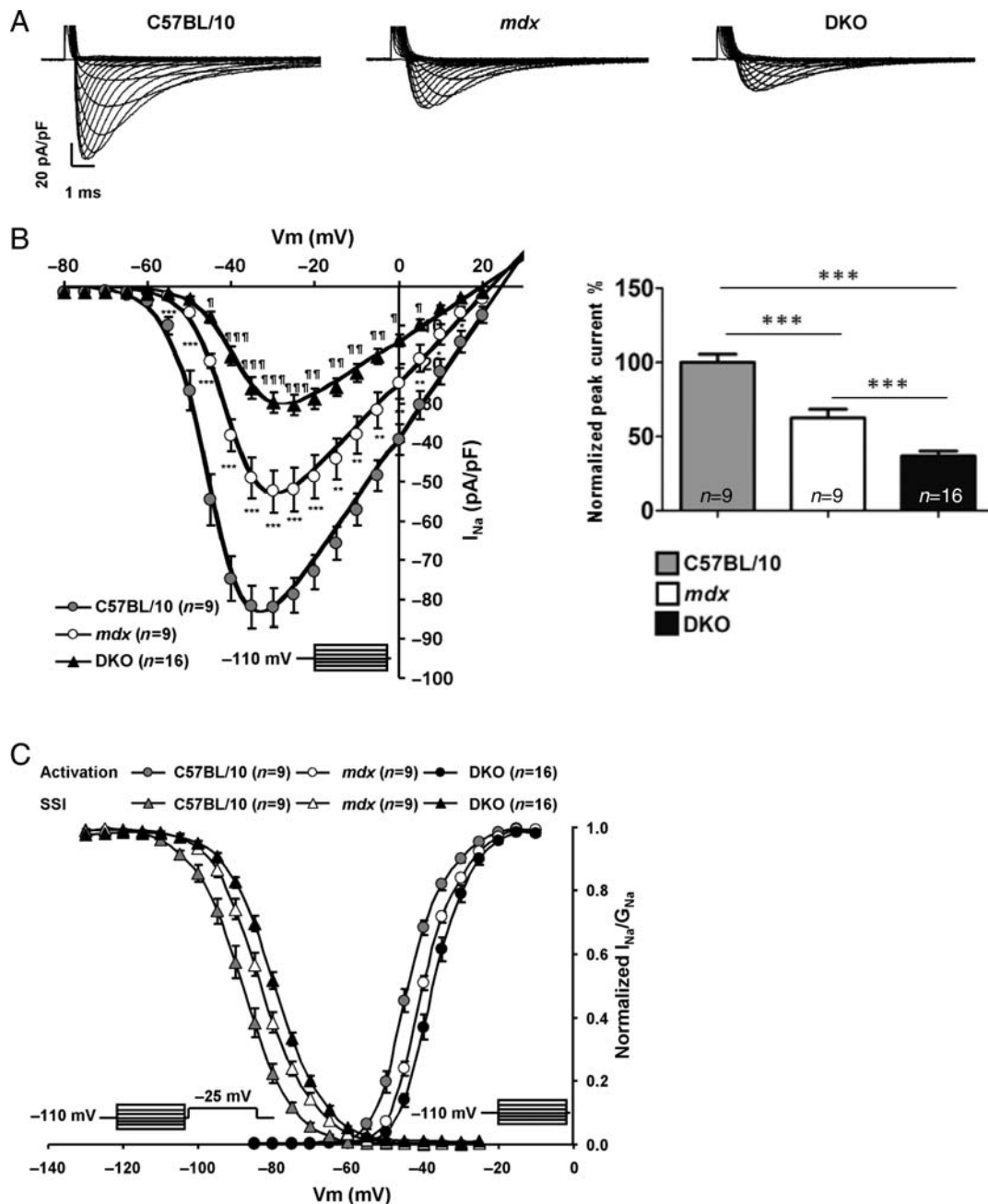
also between *mdx* and DKO ( $14 \pm 7\%$ ). The decrease in the sodium channel in ventricular lysates was accompanied by a comparable reduction ( $42 \pm 12\%$ ) in syntrophin in DKO mice compared with *mdx* mice, illustrating the consequences of disorganization of the DMC on syntrophin expression (Figure 4A and B).

### 3.4 Maximal AP upstroke velocity is reduced in DKO compared with *mdx* cardiomyocytes

Na<sub>v</sub>1.5 plays a key role in the conduction of the cardiac electrical impulse and determines the upstroke velocity ( $dV_m/dt$ ) of the cardiac AP. Considering the strong reduction in the Na<sub>v</sub>1.5 protein and current in DKO compared with *mdx* mice, we investigated whether these alterations were reflected in the APs recorded in freshly isolated ventricular myocytes from these two groups (Figure 5A). When analysing the AP upstrokes above threshold (Figure 5B), we found a significant reduction of about 30% in the maximal upstroke velocity in DKO cardiomyocytes compared with *mdx* ( $110.6 \pm 9.2$  mV/ms in DKO and  $151.8 \pm 9.4$  mV/ms in *mdx*, Figure 5C), consistent with the reduction in functional Na<sub>v</sub>1.5 protein. As shown in Figure 5D and E, no difference was found in either resting potential ( $-70.6 \pm 0.8$  and  $-72.2 \pm 0.5$  mV in DKO and *mdx*, respectively) or AP amplitude ( $118.1 \pm 2.5$  and  $120.9 \pm 2.1$  mV in DKO and *mdx*, respectively). Finally, although AP durations of 30 and 50% repolarization (APD30 and APD50) were unchanged, APD90 was slightly prolonged in DKO mice, although this difference was not significant (Figure 5F).

## 4. Discussion

In the present study, we observed that (i) utrophin indirectly binds to Na<sub>v</sub>1.5 and this interaction is dependent on the binding of the C-terminal PDZ-binding domain motif SIV of the channel to syntrophins; (ii) utrophin is up-regulated in cardiac muscle of *mdx5cv* mice; (iii) cardiac sodium channel protein levels and current densities are strongly reduced in DKO mice compared with *mdx* mice; and (iv)



loss of  $Na_v1.5$  in DKO mice leads to slowing of the cardiac AP upstroke velocity.

#### 4.1 $Na_v1.5$ and utrophin belong to the same multi-protein complex

The interaction between the cardiac voltage-gated sodium channel  $Na_v1.5$  and syntrophin proteins has been described in previous

studies.<sup>11,12</sup> We demonstrated that this interaction occurs via the PDZ domain-binding motif (SIV) of  $Na_v1.5$  and syntrophins.<sup>11</sup> There are five members ( $\alpha_1$ ,  $\beta_1$ ,  $\beta_2$ ,  $\gamma_1$ , and  $\gamma_2$ ) of the syntrophin family that are differentially distributed in skeletal muscle and other tissues.<sup>22,23</sup> In the heart, only three members have been found to be expressed,<sup>24</sup> with an unknown distribution. Gavillet et al.<sup>11</sup> observed that  $\alpha_1$ ,  $\beta_1$ , and  $\beta_2$  syntrophin may be partners of  $Na_v1.5$  *in vivo*. Syntrophins are recognized as adapter proteins, linking

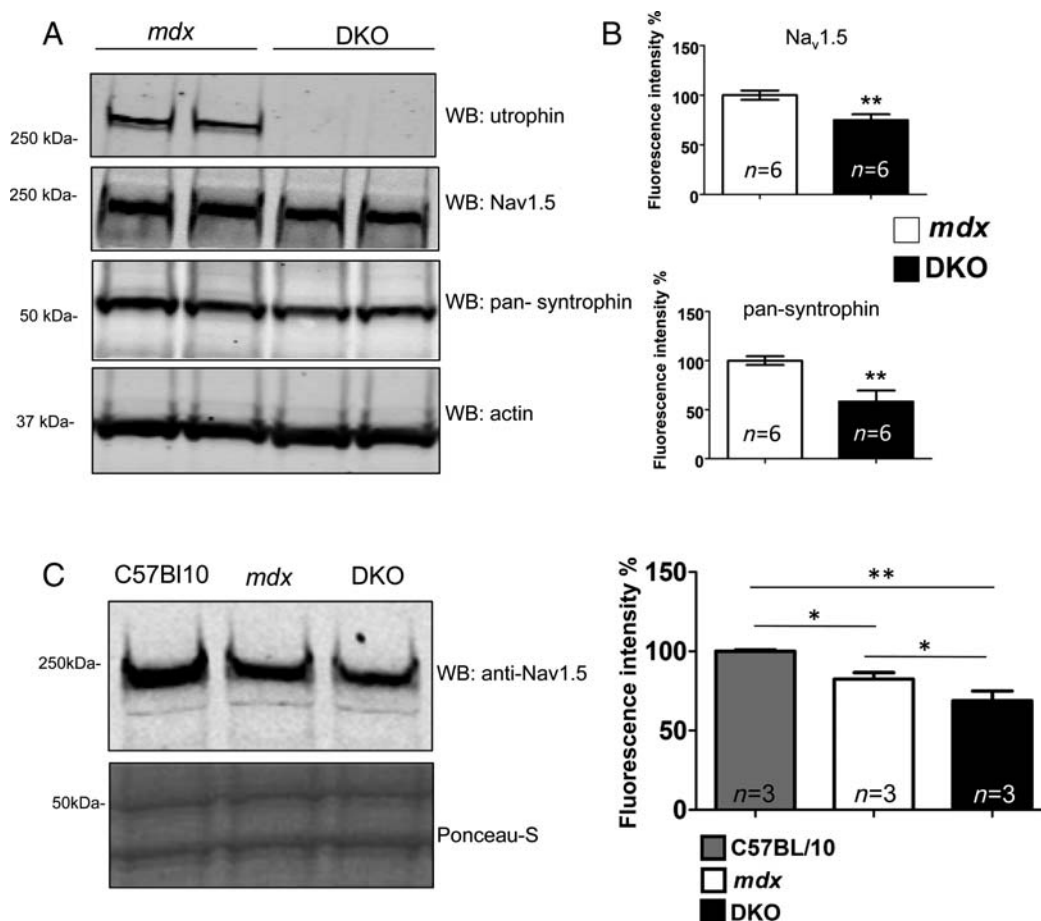
**Table 1** Voltage dependence of activation and inactivation properties of isolated ventricular cardiomyocytes from DKO, *mdx*, and C57BL/10 mice

	C57BL/10	<i>Mdx</i>	DKO
Activation	<i>n</i> = 9	<i>n</i> = 9	<i>n</i> = 16
<i>K</i> (mV)	4.9 ± 0.2	4.9 ± 0.2	4.5 ± 0.2
<i>V</i> <sub>1/2</sub> (mV)	-43.5 ± 0.7	-39.5 ± 0.4 <sup>a</sup>	-36.9 ± 0.8 <sup>b</sup>
Inactivation	<i>n</i> = 9	<i>n</i> = 9	<i>n</i> = 16
<i>K</i> (mV)	6.3 ± 0.1	6.7 ± 0.2	6.6 ± 0.2
<i>V</i> <sub>1/2</sub> (mV)	-88.2 ± 1.3	-82.9 ± 1.0 <sup>a</sup>	-79.6 ± 0.7 <sup>b</sup>

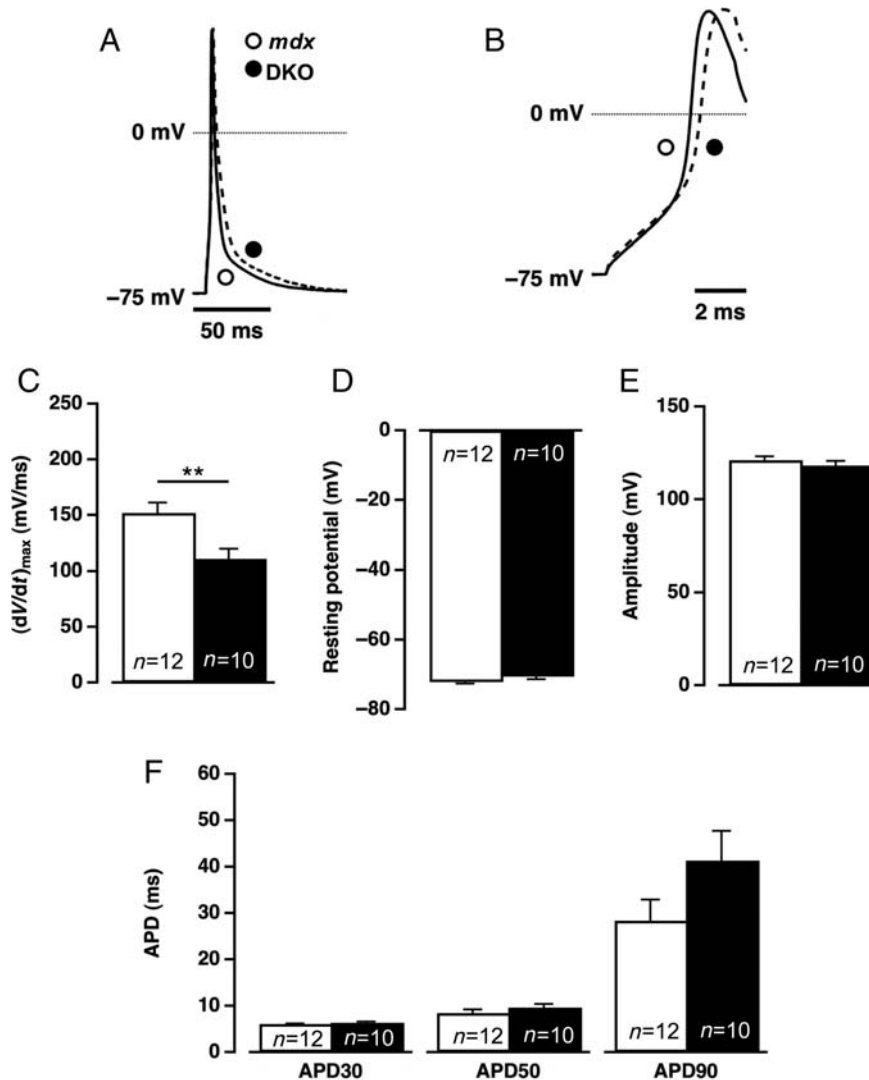
One-way ANOVA with Bonferroni post-test.

<sup>a</sup>*P* < 0.01 vs. WT mice.<sup>b</sup>*P* < 0.05 vs. *mdx* mice.

membrane and membrane-associated proteins to the DMC by binding to the C-terminus of dystrophin. The primary structures of the C-terminus of utrophin and dystrophin are very similar,<sup>25</sup> and there is evidence from *in vitro* and *in vivo* studies that utrophin can also interact with members of the DMC, such as β-dystroglycan, α-dystrobrevin-1, and syntrophins.<sup>26,27</sup> In the heart, however, evidence of an utrophin-associated complex at the cellular or subcellular level has not yet been identified. We obtained evidence that utrophin, similar to dystrophin, could be an *in vivo* partner of Na<sub>v</sub>1.5, even though we failed to demonstrate it in the mouse heart by co-immunoprecipitation experiments, despite testing many different protocols. We also showed that this indirect interaction is dependent on the direct interaction of the SIV domain of the channel with syntrophin. Although utrophin localization has been well characterized at the neuromuscular junction (NMJ),<sup>28</sup> very little is known about its cardiac subcellular localization and different studies published are controversial.<sup>29–31</sup> The generation of two different strains of



**Figure 4** Na<sub>v</sub>1.5 and syntrophin protein levels are decreased in the hearts of DKO mice. For comparison of the amounts of Na<sub>v</sub>1.5 and syntrophin present in the hearts of *mdx* and DKO mice, 40 μg of ventricular lysates was loaded on SDS–PAGE gels. The protein concentration of each lysate was measured in triplicate by Bradford assays to guarantee equivalent loading and confirmed by Ponceau coloration and western blot against actin. (A) Representative western blots performed with the indicated antibodies. (B) The amount of Na<sub>v</sub>1.5 and syntrophins was assessed by fluorescence intensity quantification with LICOR Odyssey software (*n* = 6; \*\**P* < 0.01; two-tailed Student's *t*-test). (C) Na<sub>v</sub>1.5 protein level in membrane fractions from C57BL/10, *mdx*, and DKO mice. Upper panel: representative western blot anti-Na<sub>v</sub>1.5 of membrane preparation; lower panel: Ponceau-S coloration showing a similar protein loading under different conditions. Right panel: bar graph representing quantification of Na<sub>v</sub>1.5 in membrane preparation of the different genotypes indicated. Number of mice used is indicated in the bar graph. Statistical significance was tested using a one-way ANOVA followed by a Newman–Keuls multiple comparison test (\*\**P* < 0.01 and \**P* < 0.05).



**Figure 5** AP upstroke velocity is reduced in DKO mice. (A) Representative APs recorded from the cardiomyocytes of *mdx* and DKO mice. (B) AP upstrokes from (A) displayed on expanded time-scale reveal a slowing of depolarization velocity above threshold. (C) Maximal AP upstroke velocity is reduced by ~30% in cardiomyocytes from DKO (black bar) compared with *mdx* mice (white bar). Neither resting potential (D) nor AP amplitude (E) was different between DKO and *mdx* cardiomyocytes. (F) Bar graph showing AP duration to 30, 50, and 90% repolarization in *mdx* and DKO cardiomyocytes.  $n = 12$  cells (*mdx*) and 10 cells (DKO), respectively.  $**P < 0.01$ ; two-tailed Student's *t*-test.

utrophin-deficient mice has revealed very subtle phenotypes at the NMJ,<sup>32,33</sup> without any evidence of dystrophin up-regulation. However, recent studies in vascular endothelial cells suggest that utrophin could be essential for the assembly of the DMC.<sup>34</sup> Although utrophin may play a minor role in the integrity of the DMC and the expression of functional Na<sub>v</sub>1.5 in striated muscle where dystrophin is normally abundant, our results suggest that it could be important in tissues where the composition of the DMC is altered, for instance when dystrophin is absent because mutation of its gene, or decreased in failing hearts.<sup>35</sup>

## 4.2 Utrophin up-regulation in hearts of *mdx5cv* mice

The *mdx* mouse is the most widely used animal model for DMD. The *mdx* mouse lacks dystrophin as a result of a point mutation in

exon 23 of the dystrophin gene, forming a premature stop codon.<sup>36</sup> However, 'revertant' dystrophin-positive cells have been reported in these mice.<sup>37</sup> The *mdx5cv* mouse, generated by *N*-ethyl *N*-nitrosourea mutagenesis, also lacks full length dystrophin and does not display revertant cells. Cardiac up-regulation of utrophin has been reported in different animal models lacking full length dystrophin, such as human, hamster, and the *mdx* mouse,<sup>17–19</sup> but utrophin over-expression in the hearts of *mdx5cv* mice has not been previously reported. We provide evidence that the up-regulation of utrophin also occurs in *mdx5cv* mice. In order to verify that the decrease in functional Na<sub>v</sub>1.5 found in *mdx5cv* was not strain-specific, we assessed the sodium peak current in *mdx* mice. We found a decrease similar to that previously observed in *mdx5cv*,<sup>11</sup> suggesting that down-regulation of the channel in the absence of dystrophin is a general phenomenon in mice and occurs despite utrophin over-expression.

### 4.3 Consequences of decreased Na<sub>v</sub>1.5 protein and sodium current in DKO mice

We found that deletion of the SIV motif of Na<sub>v</sub>1.5, abolishing its interaction with the syntrophin proteins, leads to a decrease in the expression of the channel in HEK293 cells. Moreover, we found that utrophin deletion in *mdx* mice leads to a strong reduction in the Na<sub>v</sub>1.5 protein and sodium peak current. A similar decrease in syntrophins was concomitantly observed, suggesting that the two proteins may belong to the same complex and have a similar fate in the absence of dystrophin or utrophin. This observation raises the question of the role of the DMC in the stabilization of sodium channels at the sarcolemma. Further investigations will be needed to determine whether targeting or anchoring of these two proteins is altered when dystrophin and utrophin are absent. DKO mice are considered to be a more appropriate model of human dystrophies than *mdx* mice as they display most of the clinical signs of DMD, including short stature, kyphosis, hindlimb weakness, laboured breathing, and premature death.<sup>36</sup> In addition, cardiac complications in DKO mice are similar to those seen in DMD patients. DKO mice exhibit abnormal ECG patterns with drastically decreased S-to-R wave ratios, a feature observed in 70–80% of the DMD patients.<sup>38,39</sup> However, conduction defects have not been reported as a prominent feature neither in DKO mice nor in DMD patients. As conduction defects are progressive in DMD<sup>40</sup> and in *SCN5A*-related animal models<sup>41,42</sup> despite an important decrease in  $I_{Na}$  from birth on, one may speculate that the age-dependent conduction defect phenotypes observed in sodium-channel-related pathologies are the result of subsequent remodelling processes involving fibrotic changes and connexin proteins remodelling as observed by van Veen *et al.*<sup>43</sup> In the present study, we found a 40% decrease in the peak sodium current in the cardiomyocytes of DKO mice when compared with *mdx* mice. Similar to results obtained by Leoni *et al.*,<sup>42</sup> we also observed that the loss of Na<sub>v</sub>1.5 proteins and current promotes significant shifts towards more positive voltages of steady-state inactivation and activation curves, increasing the fraction of sodium channels available for cardiac depolarization in DKO mice when compared with *mdx* mice. Molecular and cellular mechanisms altering the biophysical properties of Na<sub>v</sub>1.5 when channel quantity is decreased remain to be investigated. The strong reduction in the sodium current of DKO cardiomyocytes is reflected by a significant slowing of the AP maximal upstroke velocity, considered as a suitable index for the estimation of available functional sodium channels. The maximal upstroke velocity has been shown to slightly overestimate peak  $I_{Na}$  at slowed AP upstrokes,<sup>44</sup> consistent with the relative decreases in  $I_{Na}$  and  $(dV/dt)_{max}$  in DKO cardiomyocytes observed in this study. In a previous study, we have shown that the decrease of 30% in  $I_{Na}$  observed in *mdx5cv* mice compared with C57BL/6J mice is associated with an increase of 18% of the QRS complex duration in these mice.<sup>11</sup> Interestingly, these values are in very good agreement with the predictions by Shaw and Rudy<sup>45</sup> on the influence of reduced maximal sodium conductance on conduction velocity. Quantitatively, we would estimate that the reduction of another 40% in  $I_{Na}$  observed in the present study between *mdx* and DKO would result in further ventricular conduction slowing of ~15–20%. The importantly reduced  $I_{Na}$  in DKO hearts thus probably partially underlies the drastic difference in phenotype between DKO and *mdx*. However, other proteins such as inducible nitric oxide synthase or neuronal nitric oxide synthase have also been shown to be modified in the combined absence of

both dystrophin and utrophin.<sup>38</sup> Moreover, cellular pathways known to be affected in the absence of dystrophin would be expected to be more severe when utrophin is also absent. For instance, the amplification of Ca<sup>2+</sup>- and reactive oxygen species-dependent degradative pathways in dystrophin-deficient cardiomyocytes<sup>46</sup> are most likely limited by the presence and up-regulation of utrophin in *mdx* mice and would thus be expected to contribute to the drastic phenotype in DKO mice. In conclusion, the difference in phenotype between *mdx* and DKO mice is likely the sum of several cellular modifications, including alteration of the sodium current.

### 4.4 Relevance for human cardiomyopathies

Different types of cardiomyopathies are the underlying cause of death in up to 20% of individuals with DMD, a proportion that is likely to increase over the coming years.<sup>4</sup> With ventilatory support preventing respiratory-related mortality, most DMD patients will survive into their third decade, in which incidence of cardiomyopathies is nearly 100%.<sup>47</sup> We previously reported on the importance of dystrophin in the regulation of the Na<sub>v</sub>1.5 protein and its current. In this study, we demonstrate that the sodium channel phenotype observed in *mdx* mice is likely limited by the up-regulation of utrophin. This suggests that an increased up-regulation of utrophin could, in principle, restore more functional sodium channels, supporting current therapeutic strategies aimed at treating DMDs through the over-expression of endogenous utrophin.<sup>15</sup> Compared with other approaches such as gene or cell therapy that targets exogenous dystrophin expression,<sup>48</sup> pharmacological strategies designed to over-express utrophin are promising and may circumvent many obstacles inherent to gene and cell-based therapies such as adverse immune response.<sup>49,50</sup> In this study, we provide additional evidence that the over-expression of utrophin could have a positive outcome in the absence of dystrophin and could provide a means to treat cardiomyopathy in dystrophic patients by restoring the amount and integrity of proteins of the DMC, as is the case for Na<sub>v</sub>1.5.

In summary, our study provides evidence that up-regulation of utrophin in the hearts of *mdx* mice counteracts the reduction in Na<sub>v</sub>1.5 protein levels and sodium current. Utrophin deletion in these mice leads to a severe reduction in channel protein expression and to the subsequent decrease in the sodium peak current amplitude and maximal upstroke velocity of the AP. It is clear that the regulatory role of utrophin on Na<sub>v</sub>1.5 may also be mediated by syntrophin as suggested by its concomitant decrease with the sodium channel in utrophin-deficient tissue. Altogether, these findings strongly argue in favour of the development of therapeutic strategies aimed at utrophin up-regulation to treat cardiac alterations in DMD.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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